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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

We had previously reported on the purification of the α(1→3)-N-acetyl-galactosaminidase from Cl. perfringens (8000 fold) and of the α(1→3)-galactosidases, B-zyme, from Cl. sporogenes (2500 fold). Both enzymes were, nonetheless demonstrated to be still impure by polyacrylamide gel electro-

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phoresis. The purest preparation of the A-zyme was still contaminated

with related glycosidases and they even co-migrated on polyacrylamide

gel electrophoresis. This, together with other observations, implicated a

multi-enzyme complex involving SH $\rightleftharpoons$ S-S interactions.

The presence of such interactions was demonstrated in the A-zyme and

the other glycosidases, co-purifying with it, as well as with the B-zyme.

With this knowledge, it was possible to exploit these properties to purify

both the A-zyme and B-zyme.

The applicability of immuno-affinity chromatography to the purification

and separation of the exoglycosidases was also explored. A measure of

success was attained. The development of this approach was arrested by

the greater success we had by the above mentioned methods.

The action of the exo-glycosidases; $\alpha(1 \rightarrow 3)$-D-galactosidase, $\alpha(1 \rightarrow 3)$-N-

acetyl-D-galactosaminidase and $\alpha(1 \rightarrow 2)$L-fucosidase, on the blood group

substances and oligosaccharides appear to be regulated by steric hindrance.

There is a distinct sequence of cleavage observed. The A-zyme and B-zyme

must act first before the $\alpha(1 \rightarrow 2)$L-fucosidase can release the fucosyl residue.

OFFICE OF NAVAL RESEARCH

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PREPARATION OF A UNIVERSAL BLOOD DONOR TYPE

by

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ANNUAL REPORT #5

DECEMBER 1, 1979 - NOVEMBER 30, 1980

TABLE OF CONTENTS

DETAILED REPORT DECEMBER 1, 1979 - NOVEMBER 30, 1980

A. <u>PERSONNEL</u>

George N. Lowrie III, who was with the project since July 1977, has now left our group. He was responsible for the takeover of the purification of the B-zyme from Dr. Tadahisa Kogure. We had hoped to replace Mr. Lowrie with an immunologist with specific training in hybridoma methodology. Several such candidates were available on the market, but were rapidly taken up by industry.

The program limped for a while, but by parcelling out the project into smaller subunits, it was possible to utilize part-time workers to explore the effectiveness of some of our approaches.

Since then, we were able to recruit an appropriate team, and we are now forging ahead.

B. <u>A-ZYME FROM CLOSTRIDIUM PERFRINGENS</u>

1. <u>A-zyme Producing and Non-Producing Strains of Cl. perfringens</u>

The A+ and A- strains of <u>Cl</u>. <u>perfringens</u>, that we had reported on previously (1), were investigated in greater detail in order to identify the bacterial strains involved. The biochemical reactions of the two strains, Table I, as well as the fingerprints obtained from the gas chromatographic analysis, Table II, strongly implicate the A+ strains as being <u>Cl</u>. <u>perfringens</u>, and the A- strain as <u>Cl</u>. <u>sporogenes</u>. The origin of this contaminant remains an intriguing mystery, since it contains all the glycosidases found in <u>Cl</u>. <u>perfringens</u> with the A-zyme manifesting the least activity, Table III. Further work on this aspect of the work has temporarily been halted.

2. <u>Improvement in the Basic Approach to the Purification of A-zyme</u>

We had previously reported on the purification of A-zyme, which achieved

a final product that was 8000 fold pure, but with a yield of 17%, (1,2) see Table IV. We wished to build up stocks of A-zyme for the more extensive purification to be described below and so developed a quick and more effective procedure as outlined in Table V.

Possible use of acetone as a step in the fractionation was also explored. It offers an alternative approach to ammonium sulfate as a precipitin and at the same time serves as a solvent for hydrophobic membrane·components. The rationale for its use is to disrupt the possible multi-enzyme complex held together by bacterial membrane and to release the individual enzymes. The enzymes thus released would be more amenable to subsequent separation.

The ideal sequence for fractionation was found to be the introduction of the acetone fractionation step after the Sephacryl S-200, and before the DEAE step, as shown in Table VI. A comparison of ammonium sulfate and acetone fractionation procedures indicates that in both cases the recovery of enzyme is good, 98% with acetone, and 83% with ammonium sulfate.

3. Iso-electric Precipitation of A-zyme and its Effect on the Other
    Glycosidases

In our previous report (1) we had mentioned that the A-zyme could be purified by iso-electric precipitation at pH 4.5 and that it provided a useful step. It removed extraneous protein giving us an almost 3-fold purification step as well as removing other glycosidases e.g. 50% of the sialidase and β-Gal'ase, and almost 92% of the β-GlcNAc'ase.

We considered it desirable to re-examine the effect of pH on the various glycosidases. The enzyme solution contained in five different dialysis chambers was equilibrated each with a different 0.1M acetate buffer at a ratio of 1:125 for 24 hr. with one change in buffer. The buffers ranged in pH from 4.0, 4.25, 4.50, 4.75 and to 5.0. At the end of the equilibration, the precipitates that developed were removed by centrifugation

and dissolved in buffer at pH 6.3. The supernatants were likewise adjusted to pH 6.3 before determining the activities of the various glycosidases present. Table VII shows the results obtained. It is apparent that the stability of the four enzymes vary at the low pH of 4.0. The A-zyme is most stable and the sialidase the least stable. At pH 4.25, 53% of A-zyme is to be found in the precipitate, with 39-40% of the initial β-Gal'ase and β-GlcNAc'ase still remaining. At pH 5.0 the A-zyme appears to be stable, while the supernatant shows only 15 and 40% of original activity of β-Gal'ase and β-GlcNAc'ase respectively.

In view of this difference in stability of A-zyme as compared to the other glycosidases, the following experiment was performed. The enzyme preparation was dialysed against buffer at pH 5 for twice as long a period. The enzymatic activities were followed and found to be as shown in Table VIII. The A-zyme was most stable and β-Gal'ase least.

4. <u>Molecular Weight of Glycosidases from Cl. perfringens</u>

Sephacryl S-300 (73 ml) was prepared in a Pharmacia column of dimensions 2.6 x 100 cm. The column was calibrated with Dextran blue and a number of standard proteins of known molecular weight; cytochrome C 12,400, ovalbumin 45,000, bovine serum albumin 67,000 and catalase 232,000 daltons. A partially purified preparation of A-zyme was applied to the column at pH 5.0, and the 1 ml fractions collected used to determine the distribution of activities of the various enzymes:- A-zyme, β-Gal'ase, β-GlcNAc'ase by the PNP-glycoside assays and sialidase with OSM as substrate and the sialic acid released determined by the TBA method (3). The pattern of activities is shown in Fig 1.

The experiment was then repeated with the column equilibrated at pH 8.0, to give a similar pattern as at pH 5.0 for most of the enzymes with the exception of GlcNAc'ase, Fig 2.

The molecular weights computed from the elution data are shown in Table IX. While there is an appreciable error, 20%, under the conditions used. The results nonetheless indicate that most of these glycosidases have a large molecular weight and are most likely oligomeric proteins. We had previously reported 200,000 daltons for the $\alpha(1\rightarrow2)\underline{L}$-fucosidase found in $\underline{Cl}$. perfringens (4).

5. <u>Involvement of SH $\rightleftharpoons$ S-S in the Glycosidases of Cl. perfringens</u>

    a. <u>Reasons for suspecting the involvement of SH $\rightleftharpoons$ S-S</u>.

    i) Schiff (5) had previously demonstrated the need for a reducing agent, such as ascorbic acid, to reactivitate and maintain activity of A-zyme from $\underline{Cl}$. <u>welchii</u>. We have confirmed this observation and were able to achieve the same objective using DTT instead of ascorbic acid (1,2). ii) The various glycosidases persistently co-purified as a multi-enzyme complex with only an occassional hint of separation (1). iii) In our previous report (1) we had indicated that the A-zyme was inhibited by $HgCl_2$. iv) As demonstrated above, the molecular weights of the glycosidases vary from $170 - 450 \times 10^3$ daltons, which is indicative of oligomeric proteins.

    b. <u>Ability of $HgCl_2$ to inhibit all the glycosidases to varying extent</u>.

We therefore predicted that the other glycosidases of $\underline{Cl}$. perfringens would also be inhibited like the A-zyme. The ability of $HgCl_2$ to inhibit the activities of the various glycosidases is shown in Fig 3. The inhibition over the concentration range of 1×10^{-4} to 1×10^{-3} was re-determined in order to demonstrate the sensitivities of the different glycosidases to $HgCl_2$.

c. Reactivation of A-zyme inhibited with 0.5mM HgCl$_2$ by use of

DTT and EDTA

Table X demonstrates that the 0.5mM HgCl$_2$ inhibition is readily

reversed, 92%, by the lowest concentration of DTT and completely reversed,

100%, by 1.0mM DTT. EDTA at low concentration can reverse the inhibition

of A-zyme, on the other hand, at higher concentrations it inhibits the

enzyme. A mixture of the two reagents indicates that the EDTA effect

predominates suggesting that an important metal may be required for the

enzyme to function effectively.

d. Effect of a redox potential gradient on the activities of

β-Galactosidase

The activity of β-galactosidase was investigated in the presence of

varying amounts of different redox reagents;- DTT, reduced glutathione,

cysteine and oxidized glutathione. Fig 4 shows the results obtained,

indicating that DTT has the ability to enhance the activity of β-Gal'ase

whilst the other reagents inactivate it. The extent of inactivation

depends on the concentration of the reagent. Oxidized glutathione

appears to strongly inactivate the β-galactosidase, Fig 5, requiring

approximately 90% of reductant, cysteine or GSH to reactivate it. Di-

thiothreitol is effective in reversing this inactivation by GSSG starting

from a concentration of 60% upwards, Fig 5.

The effect of 40mM DTT on the various glycosidase activities was

tested on two different preparations of A-zyme. Table XI shows the results

obtained and indicates that β-GlcNAc'ase behaves differently from the A-

zyme and β-Gal'ase, showing some inactivation rather than activation in

the presence of DTT.

6. <u>The Separation of Glycosidases on Sephacryl S-200 Before and</u>
   <u>After Treatment with Dithiothreitol</u>

In the absence of DTT, the three enzymes β-Gal'ase, β-GlcNAc'ase
and A-zyme bundle together as an apparent "multi-enzyme" complex, Fig 6A.
Pretreatment with 20mM DTT followed by their filtration through an S-200
column equilibrated with DTT, at the same concentration, results in a
distinct separation of the "macromolecule" into 3 peaks, Fig 6B. Pool I,
consisting of a) large high molecular weight peak containing all 3
enzymes, and b) a similar lower molecular weight peak containing β-Gal'ase
and β-GlcNAc'ase. Pool II, with an appreciably lower molecular weight,
appears to be pure β-GlcNAc'ase. The analytical data on the starting
enzyme preparation and those recovered in the two pools, I and II, are
summarized in Table XII.

7. <u>The Separation of Glycosidases on a Covalently Immobilized Thiol</u>
   <u>Sepharose Gel</u>

An approximately 1 ml column of a covalently bound thiol gel (pharmacia)
was used for this experiment. Four ml of enzyme were applied to the
column and 0.5 ml fractions collected. The column was then washed with
buffer to remove unadsorbed material and then eluted with 10mM DTT. The
eluates were assayed for protein (Lowry) as well as for the various
enzymatic activities (PNP-glycosides). The results are shown in Fig 7.

8. <u>The Purification of α-N-Acetylgalactosaminidase by Adsorption</u>
   <u>and Elution from Mercury Phenyl Agarose Gel</u>

Mercury phenyl agarose beads (50λ) were placed at the bottom of a
tube and a solution of the A-zyme preparation in 0.05M acetate buffer
pH 5.0 was added in 500λ aliquots and agitated with the beads for a
period of 10 min. This was centrifuged rapidly and the supernate removed

for the assay of A-zyme activity and protein content. Successive 500λ

batches of A-zyme solution were added, repeating the process until it

became apparent that the gel was saturated with the A-zyme. The gel

was then rinsed with two batches of 500λ of buffer. Elution of enzymes

was then initiated using progressively increasing concentrations of

$HgCl_2$ as shown in Table XIII. The inhibiting effect of $HgCl_2$ on the

A-zyme was overcome by the addition of excess DTT.

A sample of eluate, fraction #6 Table XIII was subjected to poly-

acrylamide gel electrophoresis and stained for protein by the Coomassie

staining method, while the A-zyme and β-Gal'ase activities were detected

by staining with the appropriate PNP-glycosides (1). A sample of the crude

enzyme prior to the mercury phenyl agarose column was also run in parallel.

The results, Table XIV, indicate that most of the protein contaminants

have been removed. All of the three β-galactosidase isozymes present in

the preparation are still detectable in the mercury phenyl agarose eluate.

On the other hand one of the two isozymes of the A-zyme had been lost

in the process.

C. B-ZYME FROM CLOSTRIDIUM SPOROGENES (MAEBASHI)

1. Involvement of SH ⇌ S-S in the B-zyme from <u>Cl. sporogenes</u>

The assay for the B-zyme is a two step assay, involving the release of

galactose and then the quantitation of the galactose released with galactose-

dehydrogenase, GDH. We, therefore, first checked on the effect of $HgCl_2$

and DTT on the GDH assay, Table XV.

Having demonstrated that the inhibition of GDH by $HgCl_2$ can be reversed

by excess DTT we then tested the effect of $HgCl_2$ on the B-zyme activity

itself. The B-zyme was inactivated by the $HgCl_2$, Table XVI. This in-

activation, in contrast to that of A-zyme, apparently could not be reversed

by DTT or DTT plus EDTA. The problem was not with the galactose-dehydrogenase, since subsequent addition of galactose to the incubation mixture could be quantitatively accounted for by the GDH assay, Table XVI.

2. <u>Purification of B-zyme by the Use of a Thiol-Sepharose Column</u>.

Since the B-zyme appeared to be irreversibly inactivated by $HgCl_2$, we did not anticipate success with the use of a mercury phenyl column. We therefore explored the possible use of a thiol-bound-sepharose column. B-zyme prepared as previously described (1) was used in these studies. A solution of the B-zyme was passed through a column of covalently bound thiol-sepharose. Most of the activity (96%) came through unadsorbed. The elution pattern is shown in Fig 8. Contents of tubes 3-7 were pooled and concentrated with simultaneous dialysis in an Amicon dialyzer with an XM50 filter. Since there was some loss of activity, the diffusate was also examined and indeed was found to contain 17% of the activity, Table XVII.

Application of the indiffusible material to a Sephacryl S-200 column now showed a clear separation of B-zyme activity from the contaminating (280 nm absorbing) protein, Fig 9. This treatment resulted in an overall 2.5 fold purification and represents a significant step forward.

D. IMMUNOAFFINITY CHROMATOGRAPHY

1. As Applied to Enzymes from <u>Clostridium</u> <u>perfringens</u>

a) <u>Antibodies to an isozyme of β-galactosidase</u>

We have repeatedly observed a number of isozymes of β-Gal'ase in the various preparations of A-zyme isolated from <u>Cl</u>. <u>perfringens</u>. The slowest migrating isozyme showed no contamination with other proteins or glycosidase and was considered suitable for the production of antibodies.

Two objectives were contemplated. First, to explore the possibility of using the resulting antibody as an immuno-affinity column to remove not only the slowest but all the isozymes of β-Gal'ase from the initial preparation of A-zyme. Second, to use the antibodies generated to develop a quantitative enzyme-neutralization assay for the initiation of the hybridoma project.

The successful immunization of a rabbit with the slowest isozyme of β-Gal'ase was demonstrated by the precipitin bands obtained in double diffusion analysis in agarose, and by the ability of the antiserum to neutralize β-Gal'ase activity as measured by the PNP-β-galactoside colorimetric assay.

b) Quantitation of the Enzyme-Neutralization Assay

It has been repeatedly emphasized that the success of the hybridoma technique to separate monoclonal antibody-producing cells is dependent up-on a quick and quantitative assay to detect the antibodies produced. Since we were planning to use the hybridoma technique to isolate our glycosidases in large quantities (1) we considered it expedient to develop a sensitive assay to detect antibody-producing cells by their ability to neutralize the activity of the glycosidases.

The appearance of a publication on the production of monoclonal antibodies against β-Gal'ase from E. coli (6) put a temporary halt to these investigations. Monoclonal antibodies were isolated by these workers which showed the entire spectrum of capabilities, from the complete neutralization of the β-Gal'ase activity to that of no ability to inhibit the enzymatic activity. Some of the isolated antibodies even enhanced the enzymatic activity. The screening procedure we were developing would thus identify only one type of antibody at one end of that spectrum. It thus became apparent that it would be necessary to develop a more

comprehensive assay for screening of hybridomas. This project was therefore temporarily halted in light of the greater success we were having with the procedures involving the sulfhydryl properties of the glycosidases.

2. As Applied to B-zyme from Clostridium sporogenes

The best preparation of B-zyme, as previously described (1) still contained several contaminating proteins as demonstrated by polyacrylamide gel electrophoresis. The band with B-zyme activity, was separated from the contaminating protein bands by slicing. This further purified B-zyme was used to immunize a rabbit.

The production of antibodies to the B-zyme containing gels was demonstrated by immunodiffusion bands appearing with the rabbit post- but not pre-immunization serum. The rabbit antiserum was purified by salting out of the gamma globulin with ammonium sulfate precipitation (0-33% saturation) followed by a DEAE fractionation procedure.

The purified antibody thus obtained was then coupled to sepharose using the cyanogen bromide technique (7). Excess cyanogen bromide sites were masked by interaction with ethanolamine.

The immobilized anti-"pure"-B-zyme was now ready to use as an immuno-affinity column with a crude B-zyme preparation. The B-zyme activity readily adsorbed to the affinity column with 51% of the total protein unadsorbed, Table XVIII. A number of chaotropic reagents, namely NaSCN, urea and guanidine HCl, were used in an attempt to elute the B-zyme (8). All were successful in eluting protein from the affinity column, but the eluates were enzymatically inactive. Re-examination of the problem revealed that the chaotropic agents, at the concentrations used, 2M, inactivated the B-zyme. At a concentration of 0.2M NaSCN, the enzyme

showed almost full activity. However, when used as an eluant, the 0.2M
NaSCN eluted most of the adsorbed protein. Again, the eluate was inactive.

Resorting to a linear gradient of NaCl, 0-2M NaCl in 0.01M pH 7.0
phosphate buffer, we succeeded in accounting for 73% of the total protein
and 56% of the initial activity, and a 2-3 fold purification, Table XVIII.
This method shows promise and is worth developing.

E. PLACENTAL GLYCOSIDASES

Prof. Ray Brown from Wayne State Medical School has had extensive
experience in the separation of proteins of similar properties by the
use of iso-electric focusing. He recently undertook the challenge of
assisting us in the separation of the A-zyme from the other contaminating
glycosidases from Cl. perfringens. He experienced as much difficulty
and frustration as we had.

Recently he has switched over to working with placentas as a source
of the glycosidases. These glycosidases are of the lysosomal type, with
a pH optimum of 4.5.

Dr. Brown gave us a sample of the enzyme at the early stages of
purification. The enzyme was compared to our partially purified A-zyme
and B-zyme, Tables XIX and XX. We are eagerly awaiting more highly
purified preparations of the placental enzymes for determination of their
action on blood group substances and their effects on the viability of
erythrocytes.

F. STEREOSPECIFIC INHIBITION OF GLYCOSIDASES INVOLVED IN THE ABO BLOOD
    GROUP SYSTEM

In the previous report (1) we had indicated that only 20% of all the
fucose could be released from A^+ PSM, as compared to 100 % from H^+ PSM.

Addition of A-zyme resulted in the release of all the fucose. A clarification of the results became evident when individual oligosaccharides isolated from the initial glycoprotein by the β-elimination procedure (9), were used as substrates. The tetrasaccharide readily released all its fucose, but the pentasaccharide did not release any, Table XXI. Addition of A-zyme resulted in the cleavage of the terminal non-reducing N-acetyl-galactosaminyl residue and the molecule now became susceptible to fucosidase, Table XXI.

In like manner, the oligosaccharide alditols, obtained by the β-elimination method from B^+ ovarian cyst and used as substrate for our B-zyme assay, released 22% of its total fucose with the $\alpha(1{\to}2)$-L-fucosidase alone. The amount of fucose released by the fucosidase could be raised to 40% by the addition of B-zyme from Cl. sporogenes, itself free of fucosidase, Table XXI. Thus indicating that in this unseparated mixture of B^+ oligosaccharide alditols the terminal non-reducing α-gal-actosyl residue, the B-determined, is also effective in stereospecifically inhibiting the action of the fucosidase, The results were further confirmed by the use of chemically synthesized A- and B-active trisaccharides bound to a high molecular weight complex, Table XXII.

It is appropriate to reiterate at this point that both $\alpha(1{\to}3)$-GalNAc and $\alpha(1{\to}3)$-Gal residues inhibit the release of fucose by $\alpha(1{\to}2)$-L-fucosidase. The converse, however, is not true. $\alpha(1{\to}2)$-Fucosyl residues do not interfere in the action of A-zyme or B-zyme.

The availability of the corresponding synthetic disaccharide derivatives enabled us to establish, Table XXII, that both A-zyme and B-zyme can act on the corresponding disaccharides even in the absence of the $\alpha(1{\to}2)$-L-fucosyl residue.

ABBREVIATIONS

a-OSM	Asialo-ovine submaxillary mucin
aRBC	Asialo-erythrocytes
A-zyme	$(\alpha 1 \rightarrow 3)$ $\underline{N}$-acetyl-$\underline{D}$-galactosaminidase, enzyme that destroys A activity
β-Gal'ase	β-galactosidase
β-GlcNAc'ase	β-$\underline{N}$-acetylglucosaminidase
B-zyme	$(\alpha 1 \rightarrow 3)$ $\underline{D}$-galactosidase, enzyme that destroys B activity
CPD	Citrate Phosphate Dextrose
DTT	Dithiolthreitol
EDTA	Ethylene diamine tetra-acetate
GalNAc	$\underline{N}$-acetyl-$\underline{D}$-galactosamine
GDH	Galactose Dehydrogenase
GSH	Reduced Glutathione
GSSG	Oxidized Glutathione
H^{+}-PSM	H-active porcine submaxillary mucin
OSM	Ovine Submaxillary mucin
PAGE	Polyacrylamide gel electrophoresis
PNP	Paranitrophenyl-
R_e	Relative electrophoretic mobility
SDS	Sodium dodecyl sulfate
TBA	Thiobarbituric Acid

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    the Action of Glycosidases and Galactose Oxidase (in preparation)

LIST OF FIGURES

LIST OF TABLES

19

Fig 1

ELUTION PATTERN OF GLYCOSIDASES FROM SEPHACRYL S-300, AT pH 5.0 AND WITHOUT DTT

Fig 2

ELUTION PATTERN OF GLYCOSIDASES FROM SEPHACRYL S-300

AT PH 8.0 AND WITHOUT DTT

A-ZYME
β-GAL'ASE
β-GLCNAC'ASE
SIALIDASE

ABSORBANCE AT 405 NM

FRACTION NO.

FIG 3

EFFECT OF Hg^{++} ON ACTIVITY OF GLYCOSIDASES

A-ZYME
β-GAL'ASE
β-GlcNAc'ASE

% OF UNINHIBITED ACTIVITY

% OF UNINHIBITED ACTIVITY

$HgCl_2$ MOLARITY

FIG 4

RESPONSE OF β-GALACTOSIDASE
TO REDOX REAGENTS

Fig 5

Activity of β-Galactosidase in the Presence of Decreasing Percentage of Oxidant GSSG (2mM) and Increasing Amounts of Reductants;-

o——o 3mM DTT
+——+ 2mM GSH
□——□ 2mM CYS

Enzyme Activity

% Reductant
% Oxidant (GSSG)
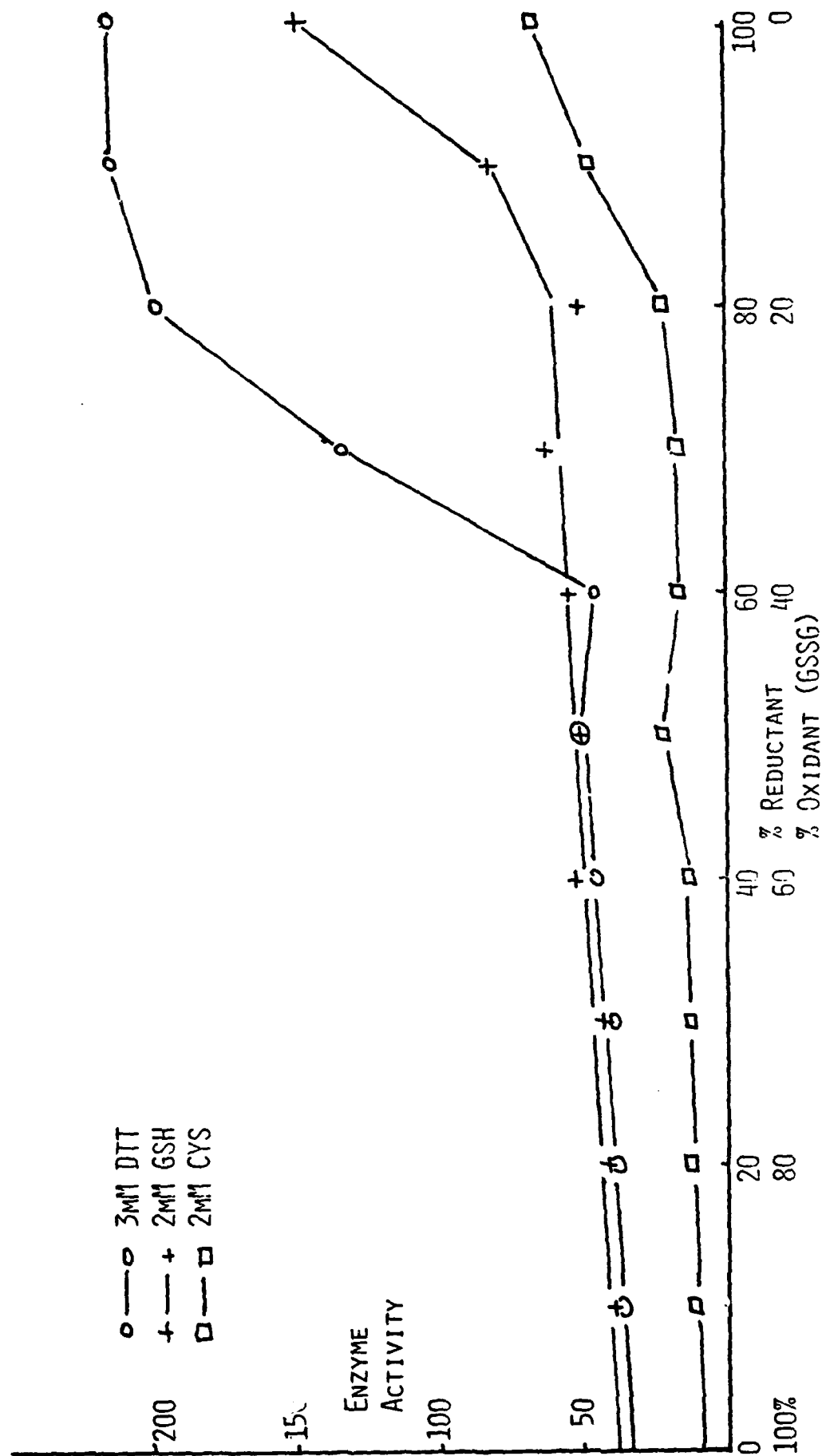
FIG 6A

SPECIFIC ACTIVITIES OF GLYCOSIDASES
SEPARATED ON SEPHACRYL S-200

●——● A-ZYME
✗——✗ β-GAL'ASE
△——△ β-GLCNAC'ASE

SPECIFIC
ACTIVITY

400

300

200

100

10   20   30   40   50   60
FRACTION VOLUME (ML)
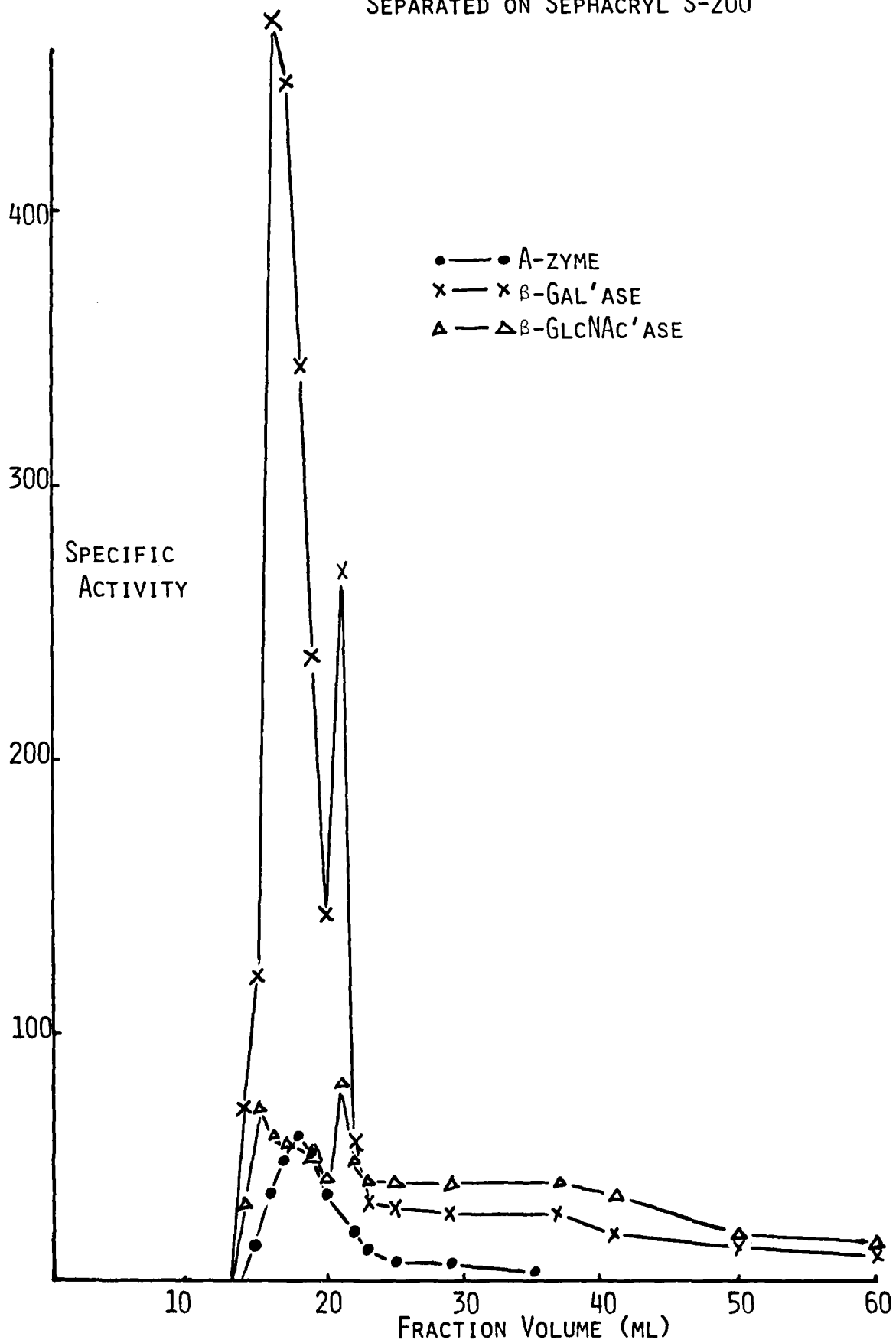
FIG 6B

SPECIFIC ACTIVITIES OF GLYCOSIDASES FROM
S-200 AFTER DTT TREATMENT (20 mM)
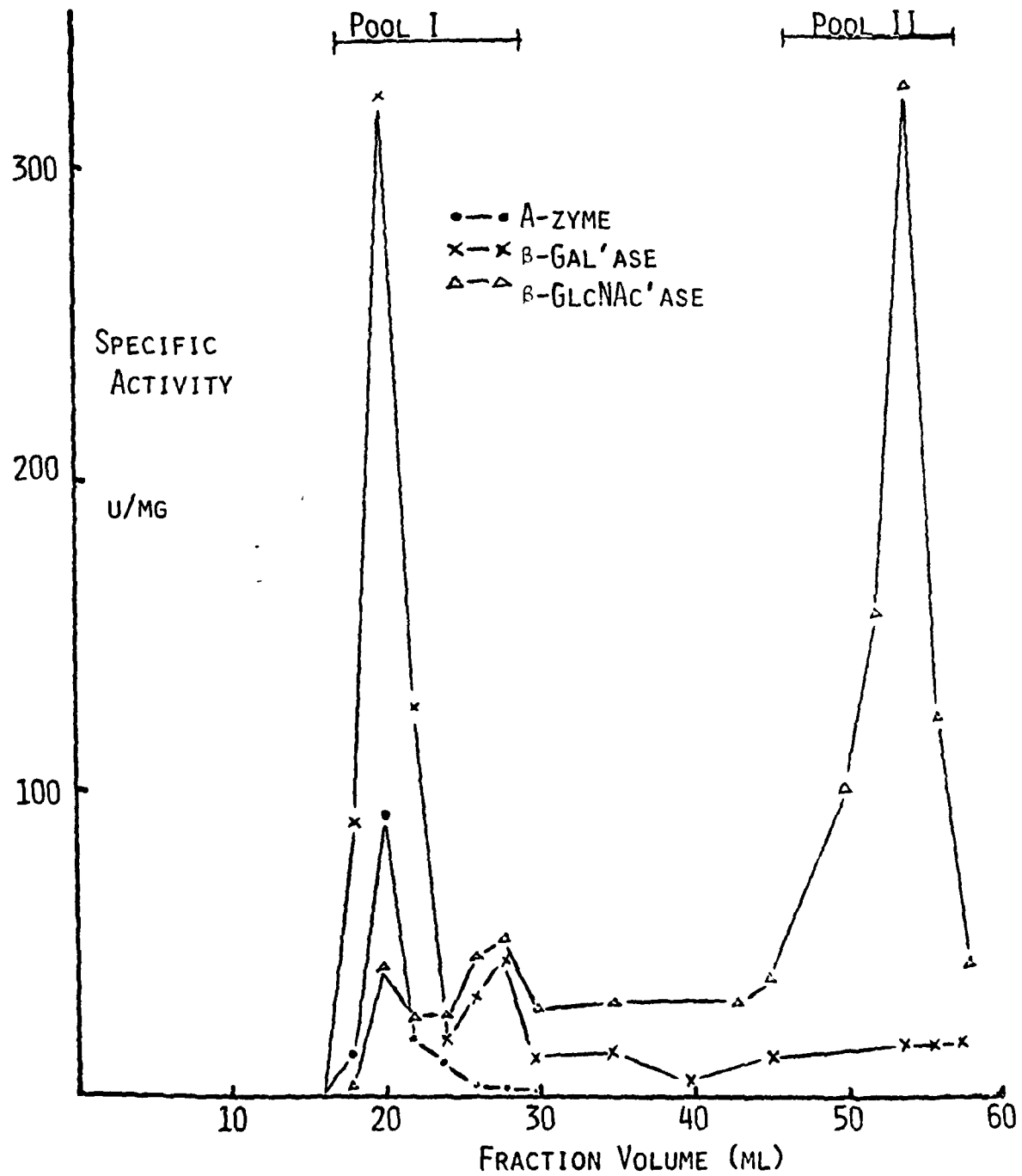
ADSORPTION AND ELUTION OF A-ZYME AND
OTHER GLYCOSIDASES FROM A COVALENT

THIOL COLUMN

A-ZYME
× β-GAL'ASE
○ β-GLcNAc'ASE
□ PROTEIN

FIG 7

ENZYME
ACTIVITY
u/ML

FRACTION No.

MG/ML
PROTEIN
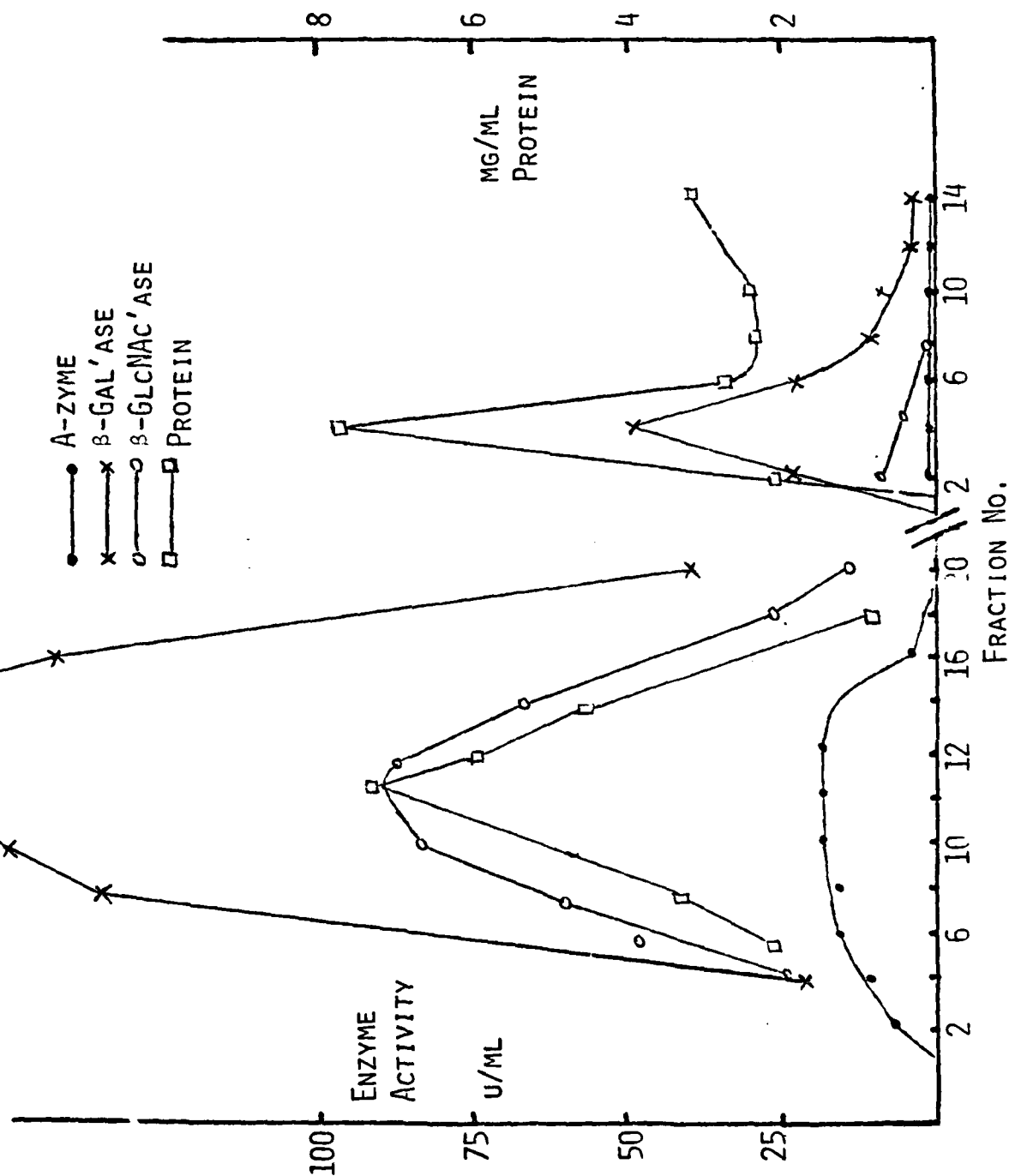
FIG 8

ELUTION PATTERN OF B-ZYME
FROM THIOL - SEPHAROSE 4B

X----X B-ZYME
ACTIVITY
A340 NM

•——• PROTEIN
A280 NM

POOLED

TUBE NUMBER (1ML)
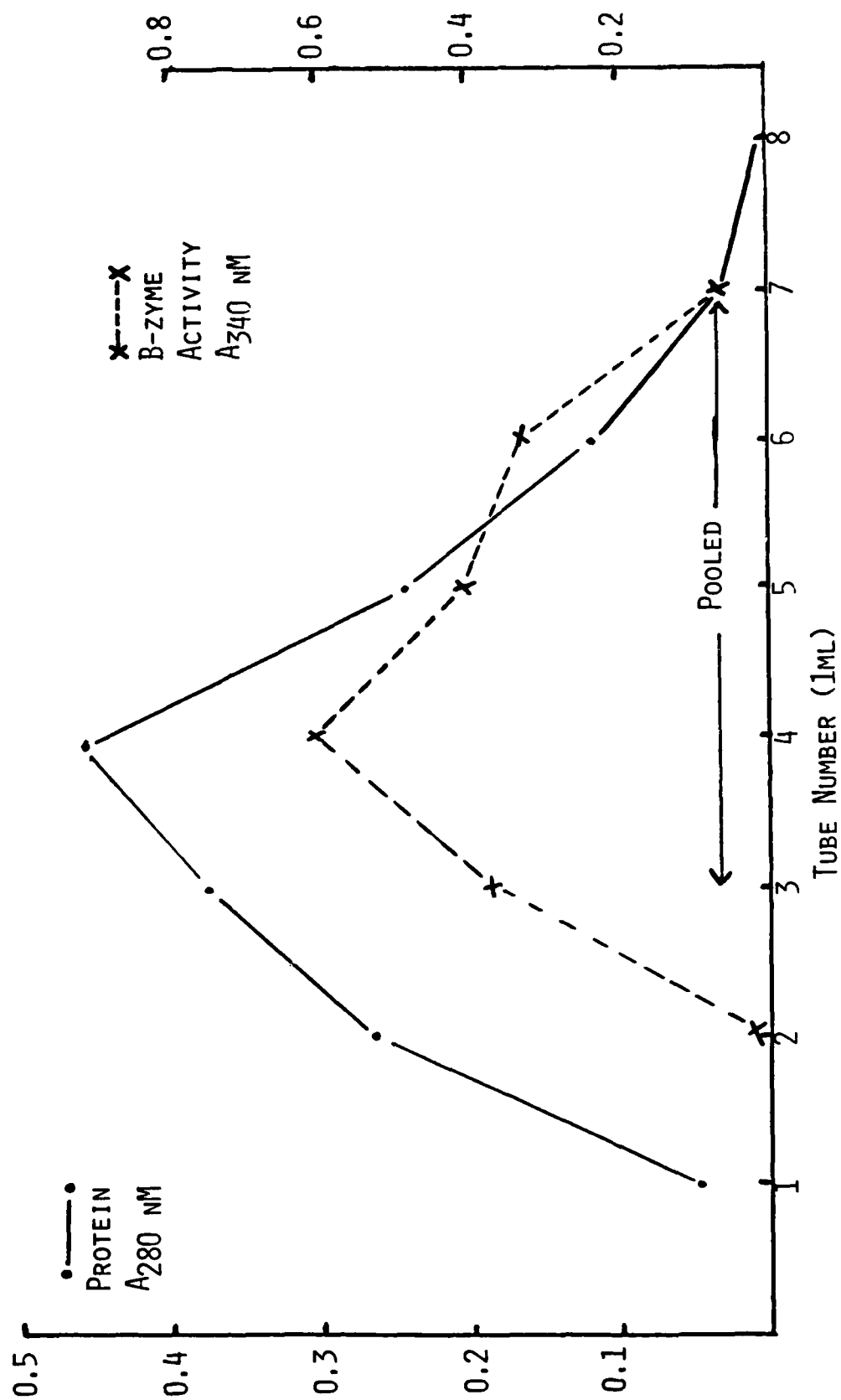
Fig 9

Sephacryl S-200

Elution Pattern of B-zyme After

Thiol - Sepharose Treatment
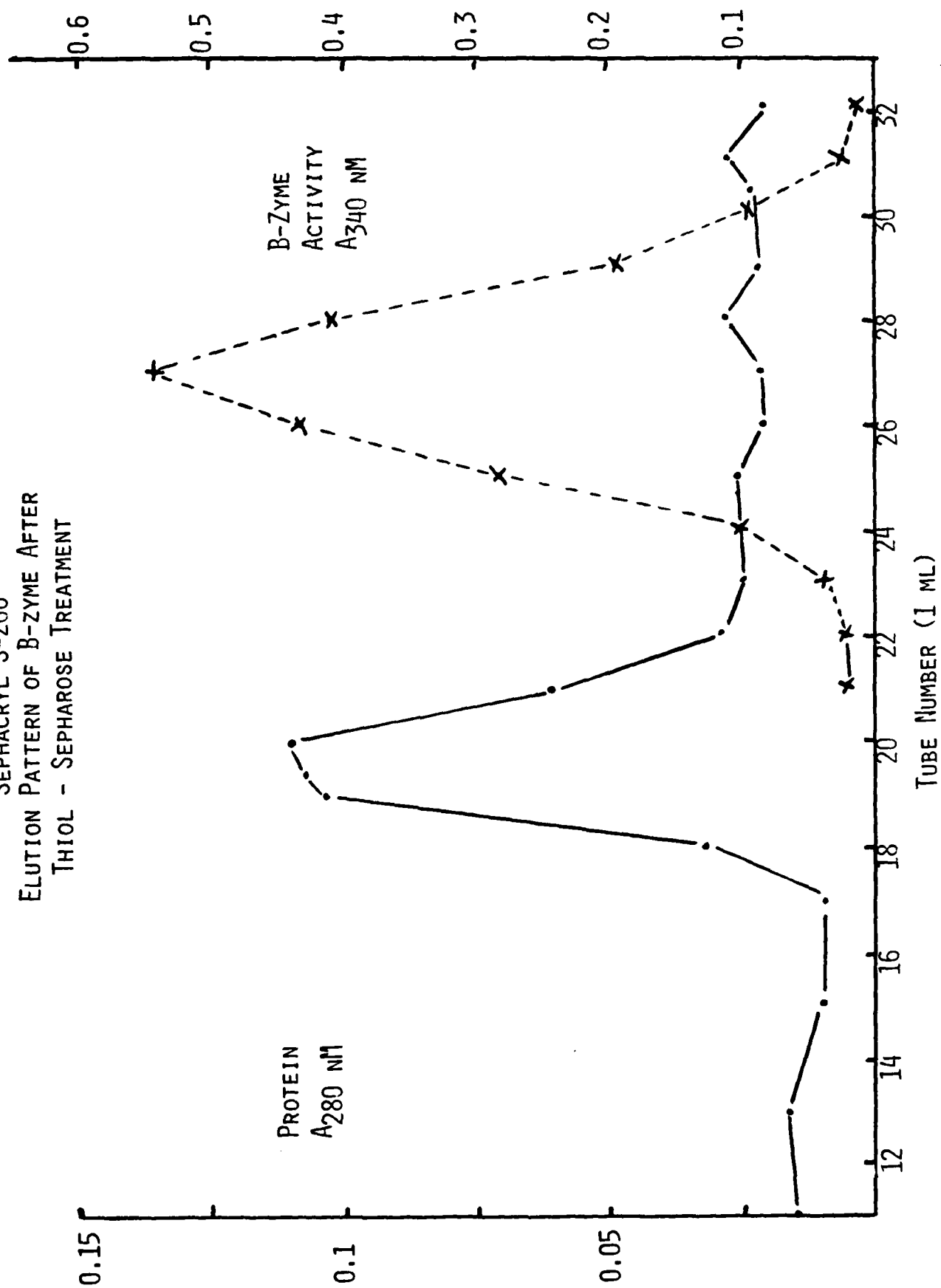
Table I

Biochemical Reactions of A^+, A^-

	A^+	A^-	PERFR.	SPOROG.
NITRATE	+	-	+	-
INDOLE	-	-	-	-
UREA	-	-	-	-
GLUCOSE	A	A	A	V
MANNITOL	A	-	-/+	-
LACTOSE	A	-	A	-
SACHAROSE	A	-	A	-
MALTOSE	A	A	A	V
SALICIN	A	-	-/+	-
XYLOSE	-	-	-	-
ARABINOSE	-	-	-	-
GELATIN	+	+	+	+
ESCULIN	-	+	-/+	+
GLYCEROL	-	-	V	-
CELLOBIOSE	-	-	-	-
MANNOSE	-	-	A	-
MELEZITOSE	-	-	-	-
RAFFINOSE	A	-	-/A	-
SORBITOL	-	-	-	-
RHAMNOSE	A	-	-	-
TREHALOSE	A	-	+/A	-/+
LECITHINASE	+	-	+	-
LIPASE	-	+	-	+

LIST OF SYMBOLS:
+ TEST RESULTS GENERALLY POSITIVE
- TEST RESULTS GENERALLY NEGATIVE
+/- TEST RESULTS MORE OFTEN POSITIVE BUT NEGATIVES OCCUR
-/+ TEST RESULTS MORE OFTEN NEGATIVE BUT POSITIVES OCCUR
A ACID REACTION
V TEST RESULT VARIABLE

Table II

Chromatographic Analysis of
Metabolic Products of A^+, A^-

A^+	A^-	Perfr.	Sporog.
l. Acetic	l. Acetic	Acetic	Acetic
l. Butyric	s. Butyric	Butyric	Butyric
s. Propionic	s. Isobutyric	(Propionic)	(Isobutyric)
	s. Propionic		(Propionic)
	s. Isovalonic		(Isovaleric)

LIST OF SYMBOLS: l. Large
 s. Small
 ( ) Variable

TABLE III

COMPARISON OF GLYCOSIDASE ACTIVITIES OF
A+ AND A- STRAINS GROWN UNDER IDENTICAL CONDITIONS

| GLYCOSIDASE | SPECIFIC ACTIVITY | | ACTIVITIES IN A- |
	A+	A-	AS % OF A+
A-ZYME	0.15	0.0015	1
β-GAL'ASE	4.50	0.34	7.6
β-GLcNAc'ASE	3.7	0.93	25.1
SIALIDASE	96	42	42.7

Table IV

PURIFICATION OF α-N-ACETYLGALACTOSAMINIDASE (A-ZYME)

Step	Sp. Act. u/mg	Yield %	Fold Purification	A-Zyme Sialidase	A-Zyme β-Gal'ase	A-Zyme β-GlcNAc'ase
1. Crude Filtrate	0.13	100	1	0.12	0.06	0.04
2. Ammonium Sulfate 0-67% Sat.	0.48	76	3.7	0.12	0.06	0.05
3. Sephacryl S-200	3.8	61	29	0.12	0.08	0.08
4. DEAE-Sephacel	55	35	425	5.3	0.18	0.41
5. Isoelectric Precipitation	149	34	1125	9.0	2.9	4.7
6. Negative Adsorption with Type O RBC Followed by: a) Sepharose 6B b) Isoelectric Pptn.	1043	17	8025	542	53	79

TABLE V

PURIFICATION OF A-ZYME BY RAPID/EFFICIENT PROCEDURE

STEP	VOL ML.	UNITS U/ML	SPECIFIC ACTIVITY U/MG	YIELD %	FOLD PURIFICATION
CRUDE FILTRATE	6640	0.35	0.025	100	1
MILLIPORE CONC.	191	12.1	0.22	100	8.8
40-65% AmSO$_4$	38.5	53	0.88	87	35
SEPHACRYL S-200	255	6.7	3.9	85	160
DEAE	100	16	30.0	70	1200

	$\frac{A\text{-}ZYME}{SIALIDASE}$	$\frac{A\text{-}ZYME}{\beta\ GAL'ASE}$	$\frac{A\text{-}ZYME}{\beta\ GLCNAC'ASE}$
DEAE	19.5	0.57	1.1

TABLE VI

USE OF ACETONE TO PURIFY THE A-ZYME
(PURIFICATION FACTOR AT EACH STEP SHOWN IN PARENTHESIS)

SEPHACRYL S-200 (1)

ACETONE (3.5)   AMMONIUM SULFATE (2.1)   DEAE (28)

DEAE (15)   DEAE (20)   ACETONE (1.6)   AMMONIUM
                                                         SULFATE (0.7)

| FINAL PURIFICATION FACTOR | 55 | 42 | 43 | 21 |
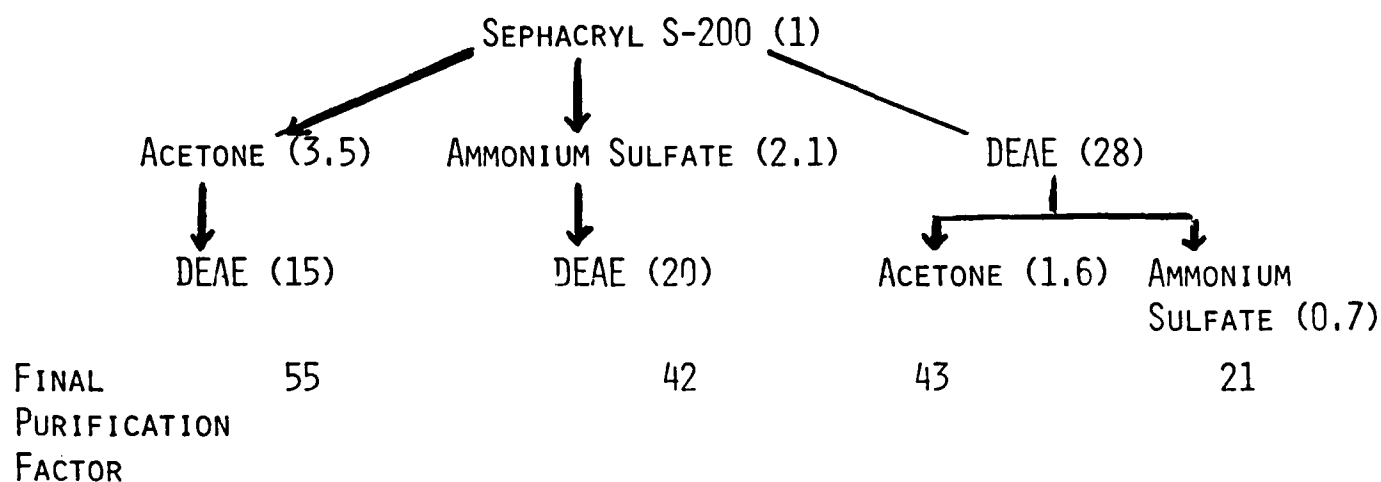
TABLE VII

EFFECT OF pH ON SOLUBILITY AND STABILITY OF BACTERIAL A-ZYME, β GALACTOSIDASE AND β NACETYLGLUCOSAMINIDASE

ENZYME	FRACTION	4.0	4.25	4.50	4.75	5.0
	% IN SUPERNATANT	46	22	50	92	107
A ZYME	% IN PPT.	14	53	36	7.4	2.9
	% RECOVERED	60	75	86	100	110
	% IN SUPERNATANT	11	5.8	17	33	40
β GLCNAc'ASE	% IN PPT.	18	39	36	21	6.2
	% RECOVERED	29	45	53	54	46
	% IN SUPERNATANT	5.6	2.1	3.4	8.5	15
β GAL'ASE	% IN PPT.	27	40	39	26	3
	% RECOVERED	33	42	42	34	18
	% IN SUPERNATANT	0	5.6	42	58	78
SIALIDASE	% IN PPT.	0	5.6	5	3	4
	% RECOVERED	0	11.0	47	61	82

Table VIII

Repeated Treatment of Glycosidases at pH 5.0 and its Effect on Activities of A-zyme, β-NAcetylglucosaminidase and β-Galactosidase

| | | % ACTIVITY | |
| | | OF SUPERNATANT RELATIVE TO UNTREATED ENZYME | |
	Untreated	1st Exposure to pH 5.0	2nd Exposure to pH 5.0
A-zyme	100	87±2	86±2
β-GlcNAc'ase	100	77±3	55±5
β-Gal'ase	100	37±1	36±4

TABLE IX

MOLECULAR WEIGHTS OF SOME GLYCOSIDASES
FROM CLOSTRIDIUM PERFRINGENS FILTRATES

ENZYME	APPROXIMATE MOL. WT. AT pH 5.0
A-ZYME	220,000
β-GAL'ASE	230,000
β-GLCNAC'ASE	170,000 – 450,000
SIALIDASE	50 – 100,000

TABLE X

Reactivation of A-zyme by DTT and EDTA
After Inhibition by 0.5 mM $HgCl_2$

I	DTT	EDTA	DTT & EDTA
.02M	100	21	23
.001M	100	46	39
.0005M	98	40	21
.00025M	92	43	41

TABLE XI

EFFECT OF DITHIOTHREITOL (DTT) ON THE GLYCOSIDASES

| | UNITS/ML | | |
	ABSENCE OF DTT	PRESENCE OF DTT*	%Δ
ENZYME PREP. I			
α-GALNAc'ASE	17.0	18.0±0.1	5.9±0.6
β-GAL'ASE	64.6±0.3	71.8±0.2	11.0±0.8
β-GLcNAc'ASE	9.7±0.2	8.7±0.1	-10.0±1.0
ENZYME PREP. II			
β-GALNAc'ASE	16.2±0.1	19.3	19.0
β-GAL'ASE	132.0±3.0	149.0±1.0	13.0±3
β-GLcNAc'ASE	34.0±0.4	31.7±0.2	-6.8±2

* 40mM DTT USED TO ASSURE MAXIMUM ACTIVITIES OF THE α-GALNAc'ASE.

Table XII

The Separation of Glycosidases on Sephacryl S-200 After Treatment with Dithiothreitol (20mM)

Enzyme	Total Units Applied	Spec. Act.	Units Recovered Pool I	Units Recovered Pool II	% Recovered Pool I	% Recovered Pool II	Spec. Act. Pool I	Spec. Act. Pool II	Purification Factor Pool I	Purification Factor Pool II
α-GalNAc'ase	40.3	32.5	39.5	0	98.0	0	43.7	0	0.1	0
β-Gal'ase	232.5	187.5	161.0	0.4	69.2	2.0	176.0	15.4	0.9	0.1
β-GlcNAc'ase	51.0	41.1	29.2	3.5	57.0	6.8	31.9	133.5	0.8	325

Table XIII

Purification of A-zyme by Adsorption and Elution from Mercury-Phenyl Agarose

Fraction	Protein mg/ml	Total Units	Specific Activity u/mg	Fold Purification
Enzyme preparation 1200 x purified	13.4	3.75	0.28	1
Elution Fractions				
1. 1mM $HgCl_2$	.17	0.12	.72	2.6
2. 1mM $HgCl_2$	.34	0.32	.95	3.4
3. 2mM $HgCl_2$	.11	0.32	2.87	10.2
4. 5mM $HgCl_2$	.08	0.31	3.84	13.7
5. 10mM $HgCl_2$	.07	0.31	4.45	16.0
6. 20mM $HgCl_2$	.05	0.32	6.48	23.1
Total		1.7		
%		45		

Enzymatic activity restored by incubation with DTT

TABLE XIV

PAGE Gels of A-zyme from
Mercury-Phenyl Agarose Column

Stain	Re Crude Enzyme		Re Eluate	
A-zyme (p-NP)	I.	.392		
	II.	.255	I.	.255
β-Gal'ase (p-NP)	I.	.413	I.	.390
	II.	.298	II.	.326
	III.	.250	III.	.255
Coomassie Blue	I.	.475	Insufficient	
	II.	.365	protein for	
	III.	.315	visible bands	
	IV.	.245		
	V.	.225		
	VI.	.205		

Table XV

Galactose Dehydrogenase Assay for Free Galactose

Incubation Mixture	% Activity
Galactose DH + Galactose	100
Galactose DH + 12.5mM DTT + Galactose	98±2
Galactose DH + 0.62mM $HgCl_2$ + Galactose	<1
Galactose DH + 1mM $HgCl_2$ + 12.5mM DTT + Galactose	100±4

Note: With galactose DH and galactose kept constant

TABLE XVI

THE B-ZYME ASSAY

INCUBATION MIXTURE	ADDITION OF EXTRA DTT TO GDH ASSAY	% ACTIVITY
ENZYME + SUBSTRATE	5mM	100
ENZYME + SUBSTRATE + 1.1mM $HgCl_2$	5mM	<1
ENZYME + SUBSTRATE + 28mM DTT	5mM	95±6
ENZYME + SUBSTRATE + 1.1mM $HgCl_2$ +29mM DTT	5mM	<1
ENZYME + SUBSTRATE + 38mM EDTA	5mM	89±2
ENZYME + SUBSTRATE + 0.57mM $HgCl_2$ +29mM DTT + 29mM EDTA		6±3

SUBSEQUENT ADDITION OF GALACTOSE IS READILY DETECTED

INCUBATION MIXTURE	GALACTOSE DETECTED
GALACTOSE	100
GALACTOSE 0.57mM $HgCl_2$ + 29mM DTT	100±4
GALACTOSE 0.57mM $HgCl_2$ + 29mM DTT + 29mM EDTA	100±1

Table XVII

Purification of B-zyme by Passage Through A Thiol-Sepharose and Sephacryl S-200 Columns

Step	Total Enz. units	% Recovery	Specific Activity	Fold Purification
1. Starting Fraction	3.3	100	8.3	1
2. Thiol-Sepharose Percolate	3.2	96	9.4	1.1
3. Amicon Filtration (XM50)	2.0	64	8.2	0.9
Diffusate	0.53	17	6.0	
4. Sephacryl S-200 - Pool of enz. active fractions	1.8	91	10.0	1.2
5. Amicon Filtration (UM2)	1.5	85	21.0	2.5
Diffusate	0.05	3	1.0	

Net recovery of purest product was 46%

Table XVIII

ADSORPTION AND ELUTION OF B-ZYME FROM AN IMMUNO-AFFINITY COLUMN
PREPARED FROM AN ANTIBODY TO PURE B-ZYME

| | ENZYME | | PROTEIN | | | |
FRACTION	TOTAL UNITS	% ACTIVITY	TOTAL	%	SPECIFIC ACTIVITY	PURIF. FACTOR
CRUDE B-ZYME	1.4	100	16	100	0.098	1
UNADSORBED PROTEIN	0	0	7.12	50.7		
B-ZYME ELUTED WITH 2M NaCl;						
A)	0.48	34	2.4	15.0	0.20	2.3
B)	0.31	22	1.2	7.5	0.26	3.0
TOTAL		56		73.2		

TABLE XIX

ACTION OF PLACENTAL GLYCOSIDASES ON ARTIFICIAL SUBSTRATES AT OPTIMUM pH 4.5

PNP-SUBSTRATE	UNITS/mL	RATIO OF ACTIVITIES
α GalNAc	5.60	1.00
α GlcNAc	0.06	0.06
β Gal	0.74	0.13
α Gal	1.20	0.21
β GalNAc	4.20	0.75
β GlcNAc	13.10	2.34

TABLE XX

COMPARISON OF PLACENTAL AND CLOSTRIDIAL GLYCOSIDASES
ON A^+ AND B^+ SUBSTANCES

1. 38-52% AM. SULF. CUT OF PLACENTAL EXTRACT OBTAINED FROM DR. RAY BROWN
2. A-ZYME OBTAINED FROM CL. PERFRINGENS
3. B-ZYME OBTAINED FROM CL. SPOROGENES

A. COMPARISON OF PLACENTAL (1) WITH BACTERIAL A-ZYME (2)

SUBSTRATE=A^+ HOG SUBMAXILLARY GLYCOPROTEIN

	SUGAR RELEASED			
	NAc HEXOSAMINE		SIALIC ACID	
	1 HR.	24. HR.	1 HR.	24 HR.
1. PLACENTAL ENZYME AT pH 4.5	0.43	1.45	0	0
2. BACTERIAL ENZYME AT pH 7.0	1.37	10.70	0.78	5.07

B. COMPARISON OF PLACENTAL (1) WITH BACTERIAL B-ZYME (3)

SUBSTRATE=OLIGOSACCHARIDE ALDITOLS OBTAINED FROM
B^+ OVARIAN CYST FLUID

	SUGAR RELEASED			
	GALACTOSE		FUCOSE	
	1 HR.	24. HR.	1. HR.	24 HR.
1. PLACENTAL ENZYME AT pH 4.5	0.36	1.07	0.41	2.5
2. BACTERIAL ENZYME AT pH 6.3	119.4	1066	0	0

TABLE XXI

ACTION OF $\alpha(1\rightarrow2)$L-FUCOSIDASE ON VARIOUS SUBSTRATES,
ALONE AND WITH OTHER GLYCOSIDASES

| | % OF TOTAL FUCOSE RELEASED FROM SUBSTRATE BY | | |
| | FUCOSIDASE ALONE FOR | PLUS 16 HR FURTHER INCUBATION WITH | |
SUBSTRATE	24 HR	A-ZYME	B-ZYME
H$^+$ PSM	98	-	-
A$^+$ PSM	21	97	-
GalNAcα3GalβGalNAcol	0	97	
$\alpha\uparrow(1\rightarrow3)$ $\uparrow(2\rightarrow6)$			
Fuc NeuNGc			
Gal β GalNAcol	100	100	-
$\alpha\uparrow(1\rightarrow3)$ $\uparrow(2\rightarrow6)$			
Fuc NeuNGc			
B$^+$ Ovarian Cyst Oligosaccharide Alditols	22	-	40

TABLE XXII

ENZYMATIC CLEAVAGE OF SYNTHETIC OLIGOSACCHARIDES

	% SUGAR RELEASED					
	α-GALNAC'ASE ALONE		α-FUC'ASE ALONE		α-FUC'ASE AND α-GALNAC'ASE	
SUBSTRATE	GALNAC	FUC	GALNAC	FUC	GALNAC	FUC
GALNAC(α1→3)GAL β→R ↑α(1,2) FUC	48	0	0	0	100	100
GALNAC(α1→3)GAL β→R	100	ND	0	ND	100	ND

	% SUGAR RELEASED					
	α-GAL'ASE ALONE		α-FUC'ASE ALONE		α-FUC'ASE AND α-GAL'ASE	
SUBSTRATE	GAL	FUC	GAL	FUC	GAL	FUC
GAL(α1→3)GAL β→R ↑α(1,2) FUC	100	0	0	0	100	100
GAL(α1→3)GAL β→R	100	ND	0	ND	100	ND

ND, NOT DETERMINED